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# The Autocrine Loop of Epidermal Growth Factor Receptor-Epidermal Growth Factor/Transforming Growth Factor-α in Malignant Rhabdoid Tumor Cell Lines: Heterogeneity of Autocrine Mechanism in TTC549

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**To investigate the effects of the autocrine loop of epidermal growth factor receptor (EGFR)-epidermal growth factor (EGF)/transforming growth factor-α (TGF-α) on the proliferation and differentiation of malignant rhabdoid tumor (MRT), we used five MRT cell lines, TM87-16, STM91-01, TTC549, TTC642, and YAM-RTK1. RT-PCR analyses revealed expression of EGFR mRNA in all MRT cell lines. In contrast, the expression of either EGF or TGF-α mRNA was detected in all MRT cell lines. Expression of EGF, TGF-α, and EGFR as determined by immunocytochemical staining and *in situ* hybridization, correlated with the results of RT-PCR. Upon differentiation-induction with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), in TTC549, showing an expression of TGF-α but not EGF initially, *de novo* expression of EGF mRNA appeared abruptly on day 2 of TPA treatment. To confirm the EGFR-EGF/TGF-α autocrine loop, we used TGF-α, EGF, and their antibodies in the cultures. Monoclonal antibody (mAb) to EGFR alone significantly inhibited the growth of cell line TTC549. However, mAb to EGF or TGF-α could inhibit proliferation of this cell line only when administrated together. Our findings would suggest that growth of the TTC549 cell line is constitutionally regulated by TGF-α/EGFR, but that inhibition of this autocrine mechanism results in transient activation of an autocrine loop involving EGF/EGFR. Our results may indicate the presence of two different autocrine loops of EGFR-EGF and/or EGFR-TGF-α in MRT cell lines. The heterogeneity of autocrine mechanisms found in MRT cell lines would be consistent with the multiphenotypic diversity and aggressive characteristics of this enigmatic tumor.**

Key words: EGF — TGF-α — Autocrine loop — Malignant rhabdoid tumor — Apoptosis

Malignant rhabdoid tumors (MRT) were first described in the kidney as a rare variant of Wilms' tumor with a 'rhabdomyosarcomatoid' pattern. MRT has an extremely poor prognosis due to a high potential for the development of distant metastases.[1)] Primary MRT has also been described in the central nervous system (CNS), pelvis, and paravertebral regions.[2–7)] A characteristic feature of MRT cells is the presence of a large eosinophilic inclusion in the cytoplasm.[8)] Various cellular origins have been proposed for MRT, including neuroectodermal,[8)] myogenic,[9, 10)] histiocytic,[11)] neural, and epithelial.[12, 13)] Recent studies with established MRT cell lines have reported multiphenotypic (neuroectodermal, mesenchymal, or both) characteristics.[14–17)]

The role of the epidermal growth factor (EGF) in the proliferation of various tumors such as human neuroblastoma has been rigorously studied.[18)] EGF accelerates *in vitro* proliferation of human fibroblasts,[19)] human hepatocytes,[20)] and human mammary epithelial cells[21)]; but it suppresses the proliferation of human epidermoid carcinoma.[22)] The production of EGF family members, such as transforming growth factor (TGF-α), by various tumor cells suggests that it may play an important role in cellular transformation. TGF-α accelerates *in vitro* proliferation of human hepatocytes,[20)] human pancreatic cancer cells[23)] and rat liver epithelial cells.[24)] It is well known that TGF-α shares a common receptor with EGF.[25)] Expression of EGF receptor (EGFR) is increased in multidrug-resistant human neuroblastoma cell lines and appears to be related to the stage of cellular differentiation in normal tissue.[26)]

Although the proliferative effects of the autocrine loop involving EGFR, EGF, and TGF-α has been investigated in a number of cancer cell lines, its role in the proliferation and differentiation of MRT cell lines has not been determined.

12-*O*-Tetradecanoylphorbol-13-acetate (TPA) is known to suppress apoptosis in many cell types, but induces apoptosis in HL-60 promyelocytic leukemia cells.[27, 28)] Our laboratory has previously reported that some MRT cell lines responded to inducers of differentiation, including γ-

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interferon, retinoic acid and TPA.[16, 17] Further, proliferation of MRT cells was significantly inhibited after treatment with TPA.

We, therefore, examined expression of EGF, TGF-α, and EGFR in MRT cell lines using RT-PCR, *in situ* hybridization, and immunocytochemistry before and after treatment with TPA. We also assessed the induction of apoptosis with TPA. In addition, we investigated the effects of the autocrine loop on tumor cell proliferation using monoclonal antibody (mAb) to EGF, TGF-α and EGFR, and in the presence of TPA as a differentiation-inducer.

## MATERIALS AND METHODS

**Cell culture and differentiation** The cell lines TM87-16, STM91-01, TTC549, and TTC642 were provided by Dr. Hiroyuki Shimada and Dr. Timothy J. Triche (Childrens Hospital Los Angeles, Los Angeles, CA). The MRT cell line YAM-RTK1 was provided by Dr. Kanji Sugita (Yamanashi Medical University, Kofu). TM87-16 was established from a pleural effusion, YAM-RTK1 from ascites and STM91-01 from the pulmonary metastasis of renal MRT. TTC549 and TTC642 were established from extrarenal primary tumors. All assays of these MRT cell lines were performed before passage 24. Clinical data on the patients whose tumors were used to establish these cell lines are summarized in Table I.

We also used the neuroblastoma cell lines TGW, NB-1, IMR-32, NH-12, and a rhabdomyosarcoma cell line RD, all originally obtained from the Health Science Research Resources Bank, Japan, as controls.

Cells were cultured in RPMI-1640 (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (ICN Biomedicals Inc., Aurora, OH). Cellular differentiation was induced using 100 n*M* TPA (Sigma, St. Louis, MO) for 6 days. Cell counts were determined and the cultured cells were used for analyzing EGF and TGF-α mRNA expression on days 0, 2, 4, and 6. Then the cells were washed 3 times with 0.1 *M* phosphate-buffered saline (PBS), and the medium was changed to TPA-free medium. Moreover, the expression of EGF and TGF-α mRNAs was examined on days 2 and 4 after changing of the medium.

**RNA preparation and RT-PCR** Total RNA from each MRT cell line was isolated using Trizol reagent (Gibco). After priming of 5 μg of total RNA with random hexadeoxynucleotide primers (TaKaRa Shuzo, Shiga), reverse transcription (RT) was performed at 42°C using MMLV Reverse Transcriptase RnaseH− (ReverTra Ace, Toyobo Co., Ltd., Osaka). Diluted RT solution was used as the template for each PCR.

**PCR primers** A forward primer (f) and a reverse primer (r) were designed using the published sequences for *EGF*,[29] *TGF-α*, and *EGFR*[30] genes. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GADPH*), was used as an internal standard.[31] All primers were obtained from Pharmacia (Tokyo).

Sequences of the primers used in this study were as follows: EGF-f (5′-GGGAAGATGACCACCACTAT-3′); EGF-r (5′-ACAAGCACCACCACGCAGAC-3′); TGF-α-f (5′-TGTTCGCTCTGGGTATTGTG-3′); TGF-α-r (5′-ACTCCTCCTCTGGGCTCTTC-3′); EGFR-f (5′-CTATGAGATGGAGGAAGACG-3′); EGFR-r (5′-CAGAGGAGGAGTATGTGTGA-3′); GAPDH-f (5′-GCCAAAAGGGTCATCATCTCTG-3′); GAPDH-r (5′-CATGCCAGTGAGCTTCCCGT-3′).

**PCR reaction** PCR amplification for a total of 35 cycles was carried out using *Taq* DNA polymerase (Toyobo) and the following PCR cycle conditions: denaturation at 94°C for 60 s, annealing for 60 s at 60°C (for EGF), or 56°C (for TGF-α and EGFR), followed by extension at 72°C for 60 s. Aliquots of PCR reaction products were electrophoresed through 2% agarose gels (Nacalai Tesque, Kyoto) containing 0.2 mg/ml ethidium bromide (Sigma).

**Competitive PCR** cDNA templates were made using a competitive DNA construction kit (TaKaRa Shuzo). Analyses were routinely performed using fixed quantities of target templates and three different quantities of competitor templates. Aliquots of PCR (10 μl) were electrophoresed through 2% agarose gels. The amount of DNA in each band was determined by quantifying the fluorescence intensity with an AIC Epi-Light UV FA1100 (Aisin Cosmos R&D, Tokyo) and the accompanying software, Luminous Imager (Aisin Cosmos). The density of each band representing amplified product from sample and competitor was measured. Then the ratios of the densities (sample/

Table I. Clinical Data on the Patients Whose Tumors Were Used to Establish the MRT Cell Lines

| Cell line | Age (months) | Sex | Primary site | Outcome | Origin of cell line |
|---|---|---|---|---|---|
| TM87-16 | 21 | M | Retroperitoneal | Died | Pleural effusion |
| STM91-01 | 8 | M | Left kidney | Died | Lung metastasis |
| TTC549 | 6 | F | Hepatic mass | Died | Primary site |
| TTC642 | 5 | F | Neck mass | Died | Primary site |
| YAM-RTK1 | 7 | M | Left kidney | Died | Ascites |

competitor) were calculated and normalized relative to GAPDH.

**Sequencing of PCR products** PCR-amplified products were directly sequenced on an ABI Prism 310 Genetic Analyzer using a "DYEnamic" ET terminator cycle sequencing kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) to confirm the sequences of PCR products.

**TUNEL assay** Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was examined with an *in situ* cell death detection kit (Boehringer Mannheim, Mannheim, Germany).

**DNA ladders** For genomic DNA isolation from $10^5$ cells of MRT cell lines, a "Track" DNA ladder isolation kit (Oncogene Research Products, Cambridge, MA) was used.

***In situ* hybridization** The PCR products of EGF and TGF-α were cloned into pGEM-4Z (Promega, Madison, WI) in two directions to obtain sense and antisense probes. These recombinant plasmids were linearized, and *in vitro* transcription was carried out in the presence of digoxigenin (DIG)-uridine 5′-triphosphate, using a T7/SP6 RNA labeling kit (Boehringer Mannheim). The *in situ* hybridization was performed as described in the literature.[32)]

**Immunocytochemistry** Cells were rinsed and fixed with 4% paraformaldehyde, 0.5% glutaraldehyde, and 0.2% picric acid in 0.1 *M* PBS. Immunocytochemical staining was performed using a 1:100 dilution of the mouse anti-human EGFR-1 (Oncogene Research Products). Slides were then incubated with peroxidase-conjugated anti-mouse immunoglobulin (Envision+, Dako, Co., Carpinteria, CA) for 30 min. Reaction products were visualized with 0.05% diaminobenzidine solution containing 0.01% hydrogen peroxide for 5 min at room temperature. mAb to EGFR-1 of mouse (Oncogene Research Products) (1:100) was used as a primary antibody with nonimmune mouse IgG (Oncogene Research Products) as a negative control. Double-stain immunocytochemistry was also performed by incubating the fixed cells overnight at 4°C with rabbit anti-EGF antibody (1:100) (Oncogene Research Products) and mouse anti-TGF-α antibody (1:100) (Oncogene Research Products), followed by incubation with rhodamine-conjugated goat anti-rabbit IgG (Cappel, Durham, NC) and FITC-conjugated sheep anti-mouse IgG (Cappel) for 2 h. All slides were then observed under a laser scanning confocal fluorescence microscope (BioRad MRC 600, Bio-Rad Microscience Ltd., Hemel Hempstead, UK).

**Growth promotion and inhibition studies** The MRT cells were inoculated at a density of $10^5$ cells/plate in serum-free medium (SFM) (Ajinomoto, Tokyo) containing varying concentrations of antibodies for EGF, TGF-α or EGFR. Anti-human EGF, TGF-α, EGFR-1 (which is known to inhibit EGF binding) and EGFR-2 (which does not inhibit EGF binding) were purchased from Oncogene Research Products. Appropriate amounts of nonimmune mouse IgG were added to control cultures. Cell number was determined at 2-day intervals from 0 to 6 days.

## RESULTS

**Light microscopic findings before and after differentiation-induction** Upon TPA induction, some MRT cell lines produced elongated cytoplasmic processes with multiple varicosities and cell proliferation was significantly inhibited subsequently (Fig. 1, A and B).

**RT-PCR before differentiation-induction with TPA** Expression of EGF mRNA was detected only in the cell line YAM-RTK1. All cell lines also expressed TGF-α,

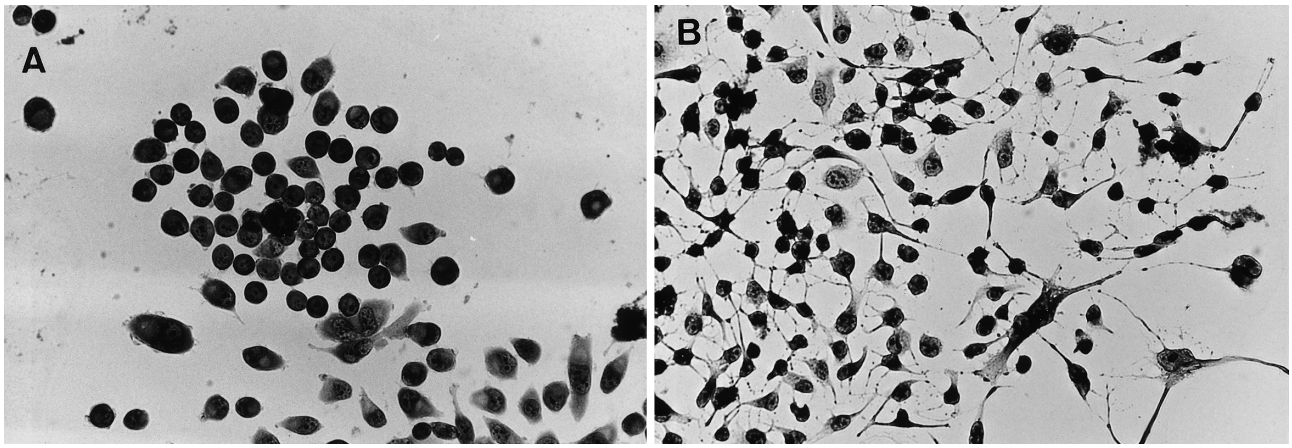


Fig. 1. TM87-16 cells before and after differentiation-induction with TPA. A. Light microscopy showing proliferation of round to polygonal cells (Giemsa, original magnification ×200). B. Light microscopy of cells treated with TPA, demonstrating *de novo* production of elongated cytoplasmic processes with multiple varicosities (Giemsa, original magnification ×200).

except YAM-RTK1. As seen in Fig. 2, we demonstrated that all 5 MRT cell lines expressed EGFR mRNA using RT-PCR.

**Analysis of apoptosis** We searched for apoptotic cells by analysis of DNA ladder formation and TUNEL assay. Amounts of TUNEL-positive cells in all MRT cells were not increased and DNA fragmentation resulting in 180–200 bp DNA ladders was not detected before or after treatment with TPA (data not shown).

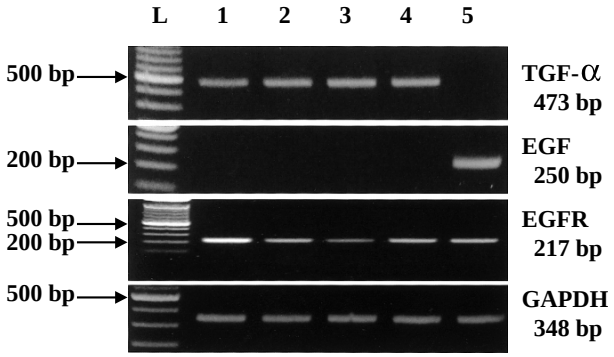


Fig. 2. RT-PCR analysis of EGF, TGF-α and EGFR mRNA expression in five MRT cell lines prior to differentiation-induction. Lane L, 100 bp DNA ladder; lane 1, TM87-16; lane 2, STM91-01; lane 3, TTC549; lane 4, TTC642; lane 5, YAM-RTK1. TGF-α was detected in TM87-16, STM91-01, TTC549 and TTC642. EGF was expressed only in YAM-RTK1. EGFR was found in all MRT cell lines.

**Competitive PCR after TPA treatment** The cell lines were induced to differentiate by treatment with TPA for 6 days, and monitored for changes in EGF and TGF-α mRNA expression using competitive PCR every 2 days from day 0 to 6. The findings with the three MRT cell lines; TM87-16, STM91-01, and TTC642 were identical. No expression of EGF mRNA was observed but TGF-α mRNA expression was decreased to half of its initial value by day 6 (Fig. 3A). We could not detect new TGF-α mRNA expression, but significant down-regulation of EGF mRNA expression was observed in YAM-RTK1 over the treatment period (Fig. 3B).

In these four cell lines, the phenomenon was reversible (data not shown). Expression of TGF-α mRNA in TTC549 was down-regulated gradually on day 2 to 6 with TPA treatment. Decrease of TGF-α was significant on day 2 and 6. EGF mRNA expression was not detected on day 0, but appeared on day 2 and then decreased gradually on day 4 to 6 of TPA treatment. The initial value of TGF-α mRNA expression and low-level expression of EGF mRNA were detected on day 2 after changing the medium to TPA-free. Expression of EGF mRNA disappeared on day 4 after changing the medium (Fig. 3C).

*De novo* expression of either EGF or TGF-α mRNA was not found after induction with TPA in any of the neuroblastoma and RD cells (Table II).

***In situ* hybridization** The results of *in situ* hybridization studies for all five MRT cell lines and control cell lines correlated with the mRNA expression detected by RT-PCR before and after treatment with TPA (data not

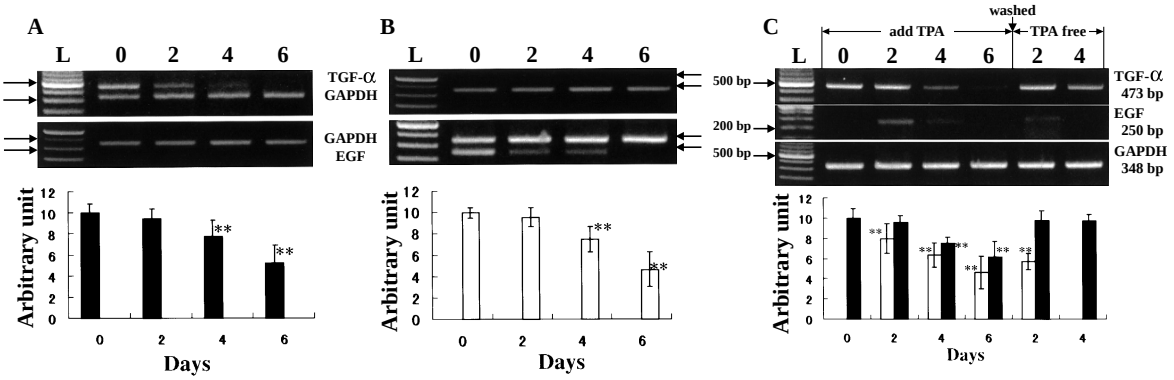


Fig. 3. Competitive PCR analysis of EGF and TGF-α mRNA expression during 6 days of differentiation-induction with TPA. Representative results from experiments with the three MRT cell lines TM87-16, STM91-01 and TTC642 (3A). Down-regulation of TGF-α was detected in three MRT cell lines. EGF down-regulation was observed in YAM-RTK1 (3B). Lane L, 100 bp DNA ladder; lane 0, day 0; lane 2, day 2; lane 4, day 4; lane 6, day 6. ** $P<0.005$ relative expression of EGF or TGF-α mRNA on day 0 versus days 4 or 6 of treatment with TPA. Expression of TGF-α mRNA was down-regulated. In contrast, EGF mRNA newly appeared on day 2 of TPA treatment in TTC549. The initial level of TGF-α mRNA expression and low expression of EGF mRNA were detected on day 2 after changing the medium to TPA-free. Expression of EGF mRNA disappeared on day 4 after changing the medium (3C). Lane L, 100 bp DNA ladder; lane 0, day 0; lane 2, day 2; lane 4, day 4; lane 6, day 6. ** $P<0.005$ relative expression of TGF-α mRNA on day 0 versus days 4 or 6 of treatment with TPA. ** $P<0.005$ relative expression of EGF mRNA on day 0 versus days 2, 4 or 6 of TPA treatment, and versus days 2 or 4 of TPA-free condition. ■ TGF-α, □ EGF.

Table II. Summary of mRNA Expression in Five MRT and Four Neuroblastoma, and Rhabdomyosarcoma Cell Lines before and after Treatment with TPA

| Cell line | Before induction with TPA | | | After induction with TPA | | |
|---|---|---|---|---|---|---|
| | TGF-α | EGF | EGFR | TGF-α | EGF | EGFR |
| MRT | | | | | | |
| TM87-16 | + | − | + | ↓ | → | → |
| STM91-01 | + | − | + | ↓ | → | → |
| TTC549 | + | − | + | ↓ | ↑* | → |
| TTC642 | + | − | + | ↓ | → | → |
| YAMRT | − | + | + | → | ↓ | → |
| Controll cell line | | | | | | |
| TGW | − | + | + | → | ↓ | → |
| NB-1 | − | + | + | → | ↓ | → |
| IMR-32 | − | + | + | → | ↓ | → |
| NH-12 | + | − | + | ↓ | → | → |
| RD | + | − | + | ↓ | → | → |

+, expressed; −, not expressed; →, no change; ↑, up-regulated; ↓, down-regulated; *, expressed only after TPA induction.

shown). EGF mRNA was detected both in the cytoplasm of TTC549 cells after induction with TPA (Fig. 4).

**Immunocytochemistry** Immunocytochemical staining of cells of the five MRT cell lines using a monoclonal antibody demonstrated EGFR protein in the cytoplasm and on the plasma membrane (Fig. 5). The results of immunocytochemistry using anti-EGF or anti-TGF-α mAb correlated well with those of RT-PCR and *in situ* hybridization. TM87-16, STM91-01, and TTC642 expressed TGF-α protein, but not EGF protein prior to TPA treatment. YAMRTK1 cells showed a positive staining only for EGF protein, but not TGF-α protein before TPA treatment. In contrast, TTC549 cells expressed both TGF-α and EGF proteins, with positive staining in the cytoplasm and on the plasma membrane, after treatment with TPA (Fig. 6, A and B). In control cell lines, immunocytochemical staining corresponded with the expression of these mRNAs in all these cell lines (data not shown).

**Growth promotion and inhibition studies** To confirm the role of EGFR-EGF/TGF-α in MRT cells, we exam-

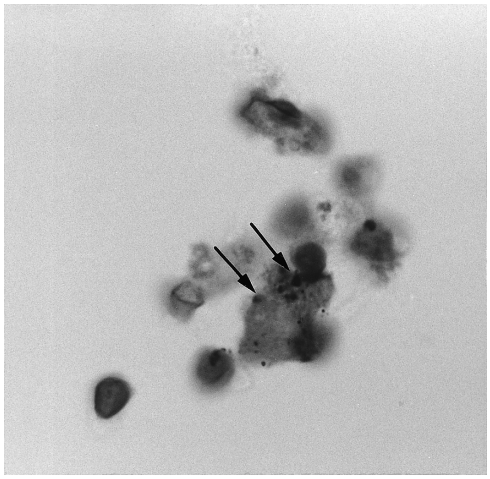

Fig. 4. *In situ* hybridization of EGF in TTC549 after induction with TPA. Hybridization signals were observed in cytoplasm on day 2 after treatment with TPA in TTC549 cells (arrows).

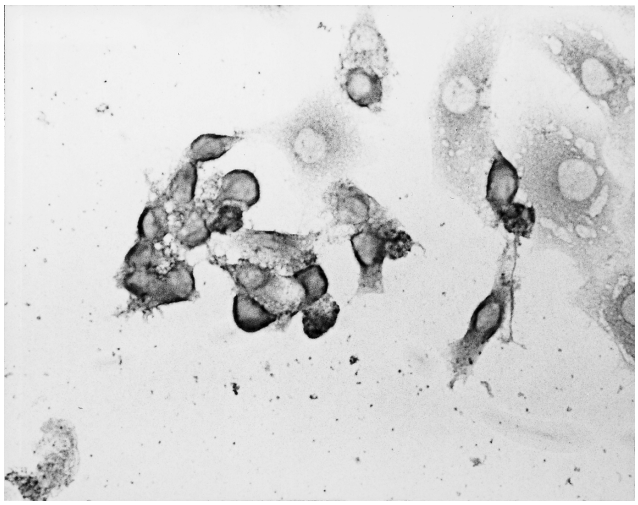

Fig. 5. Immunocytochemistry for EGFR protein in TTC549 cells. TTC549 cells showed epitheloid morphology and expressed EGFR proteins in the cytoplasm and on the plasma membrane.

ined the effects of mAbs against EGF, TGF-α and EGFR on the growth of five MRT cells. Although even up to 10 n*M* TGF-α or EGF was added to the culture medium, no significant growth stimulation was observed (data not shown). Anti-EGFR-1 mAb (which inhibits ligand binding) suppressed the growth of all MRT cell lines in a dose-dependent manner from 1 to 5 μg/ml (Fig. 7A). However, anti-EGFR-2 mAb, which does not affect ligand binding, had no effect on cell growth (Fig. 7B). Anti-TGF-α mAb significantly ($P<0.005$) reduced the cell growth of the MRT cell lines; TM87-16, STM91-01, and TTC642 in a dose-dependent manner (Fig. 8A). Anti-EGF mAb showed no growth-inhibitory effect on these three cell lines even at high concentrations of 5 μg/ml (Fig. 8B), while in YAM-RTK1, anti-EGF mAb significantly inhibited the cell growth, but anti-TGF-α mAb did not (Fig. 9, A and B).

The cell line TTC549, however, seemed to be capable of utilizing either the EGFR/TGF-α or EGFR/EGF autocrine pathway. Simultaneous administration of both anti-TGF-α and anti-EGF mAb to the culture was required to inhibit cell growth (Fig. 10).

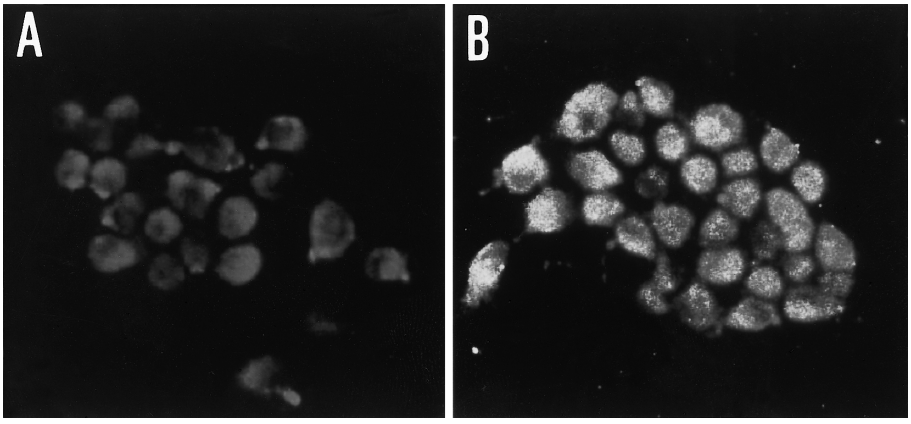


Fig. 6. Double-stain immunocytochemistry for EGF protein (rhodamine) and TGF-α protein (FITC) in TTC549 cells. TTC549 cells before differentiation-induction with TPA. Cells stained for TGF-α (red) in cytoplasm and on the plasma membrane (A). TTC549 cells after treatment with TPA. Double staining for TGF-α (red) and EGF (green) were simultaneously observed (B).

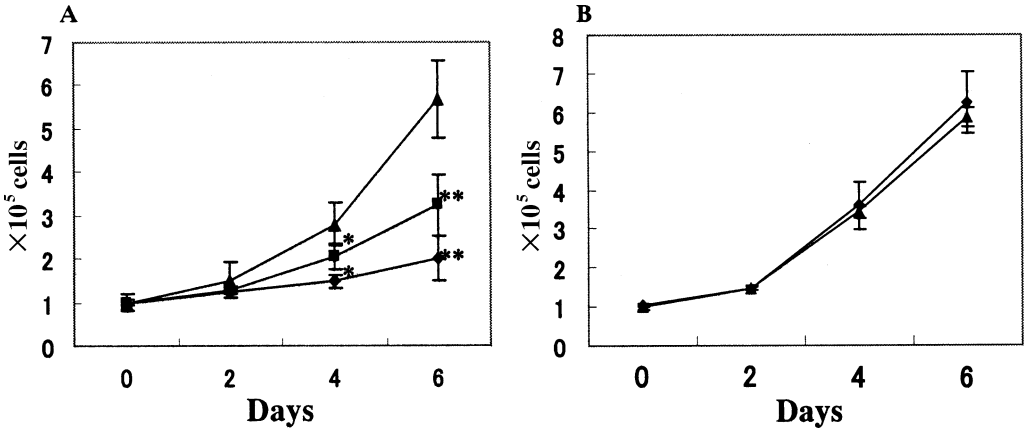


Fig. 7. Anti-EGFR-1 and -2 mAb, which inhibit ligand binding, inhibited the growth of all five MRT cell lines. Cells were seeded at a density of $10^5$ cells/plate in serum-free medium. After a 24 h incubation, the medium was changed to SFM containing 1 μg/ml (■), 5 μg/ml (◆) of anti-EGFR-1 mAb or 5 μg/ml of nonimmune mouse IgG (control) (▲) (7A). Cells were incubated in SFM containing 5 μg/ml of anti EGFR-2 mAb (◆) or 5 μg/ml of nonimmune mouse IgG (control) (▲). Cell numbers were counted at the time indicated. ** $P<0.005$ versus control cultures (7B).

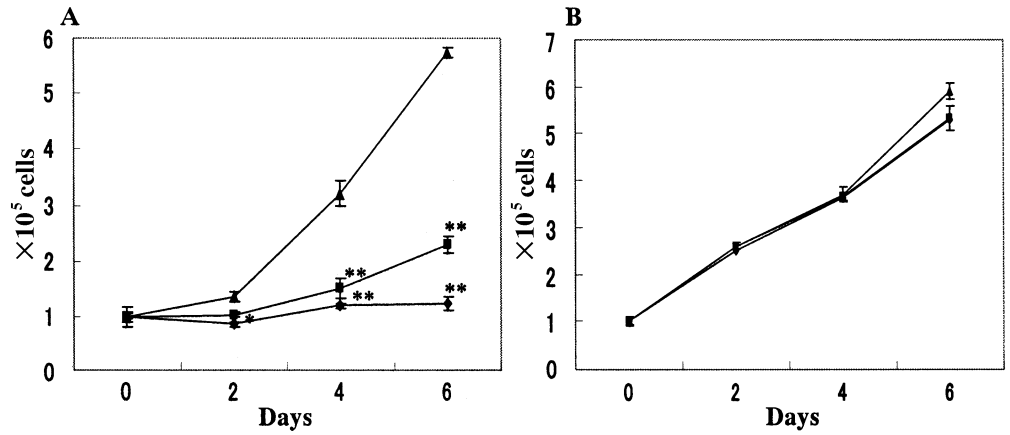


Fig. 8. Growth inhibition with anti-TGF-α or anti-EGF mAbs on TM87-16, STM91-01 and TTC642 cells. Cells were seeded at a cell density of $10^5$ cells/plate in serum-free medium. Cells were incubated in SFM containing anti-TGF-α mAb 1 μg/ml (■), 5 μg/ml (◆), or 5 μg/ml of nonimmune mouse IgG (control) (▲) (8A). Cells were incubated in SFM containing anti-EGF mAb 1 μg/ml (■), 5 μg/ml (◆), or 5 μg/ml of nonimmune mouse IgG (control) (▲) (8B). Cells were incubated for the times indicated and the number of cells was counted. * $P<0.05$, ** $P<0.005$ versus control cultures.

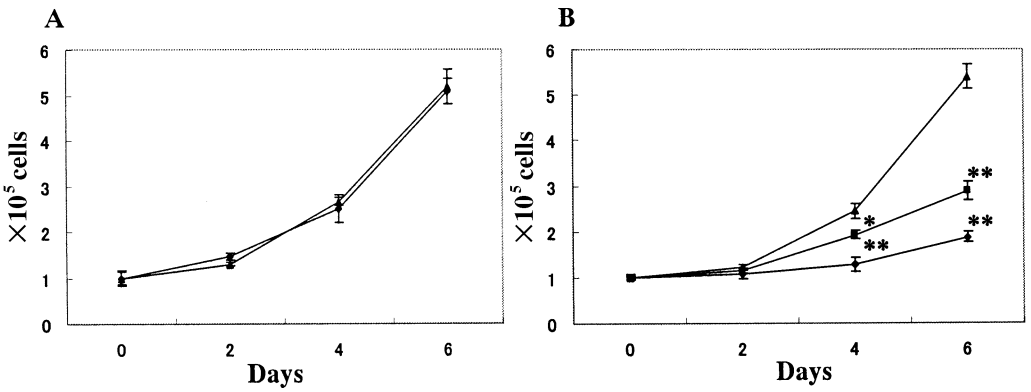


Fig. 9. Growth-inhibitory effects of anti-TGF-α and -EGF mAb on YAM-RTK1 cells. Cells were seeded at a cell density of $10^5$ cells/plate in serum-free medium. Cells were incubated in SFM containing of anti-TGF-α mAb 5 μg/ml (◆), or 5 μg/ml of nonimmune mouse IgG (control) (▲) (9A). Cells were incubated in SFM containing of anti-EGF mAb 1 μg/ml (■), 5 μg/ml (◆), or 5 μg/ml of nonimmune mouse IgG (control) (▲) (9B). Cells were incubated for the times indicated and the number of cells was counted. * $P<0.05$, ** $P<0.005$ versus control cultures.

## DISCUSSION

Both EGF and TGF-α bind to EGFR and stimulate autophosphorylation of the receptor,[25] ultimately transducing a signal for cell proliferation.[33] Coexpression of both growth factor and its receptor in the same cell is a characteristic of autocrine growth regulatory loops. Sporn and Roberts originally proposed the term 'autocrine secretion' for this self-stimulatory mechanism.[34] The autocrine loop of EGFR-EGF/TGF-α has been widely demonstrated in many kind of cancers and cancer cell lines.[35, 36] However, its biological significance in MRT remains unknown.

In this study, we examined the association of the autocrine loop of EGFR-EGF/TGF-α on the proliferation of MRT cell lines using by RT-PCR, *in situ* hybridization and double-stain immunocytochemistry. Of five MRT cell lines, there were four cell lines expressed EGFR and TGF-α mRNAs but not EGF mRNA. These four MRT cell lines are presumably regulated by a TGF-α/EGFR autocrine loop. However, one cell line, YAM-RTK1, was regulated by an EGF/EGFR autocrine loop. Three neuroblastoma cell lines showing expression of mRNA for EGF and EGFR also seemed to have an EGF/EGFR autocrine loop.[37]

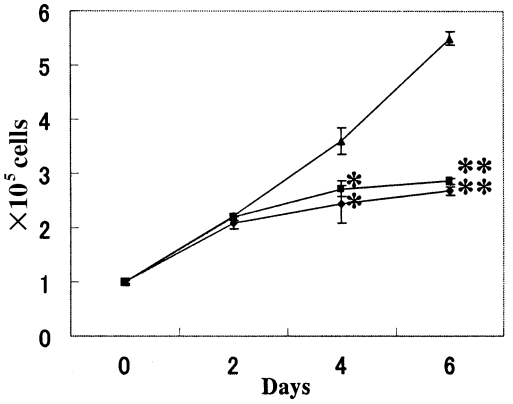


Fig. 10. Growth-inhibitory effects of anti-TGF-α and -EGF mAb on TTC549 cells. Cells were seeded at a cell density of $10^5$ cells/plate in serum-free medium. Cells were incubated in SFM containing 5 μg/ml of anti-TGF-α mAb and 1 μg/ml of anti-EGF mAb (■), 5 μg/ml of anti-TGF-α mAb and 5 μg/ml of anti-EGF mAb (◆), or 5 μg/ml of nonimmune mouse IgG (control) (▲). Cell counts were performed up to day 6. * $P<0.05$, ** $P<0.005$ versus control cultures.

Apoptotic cells were not increased after treatment with TPA. Cell proliferation was inhibited by TPA, but this phenomenon was reversible. These results suggested that the autocrine loop of EGFR-EGF/TGF-α was inhibited transiently by TPA. We, therefore, examined the effects of differentiation-induction with TPA on EGF or TGF-α mRNA expression using competitive PCR, *in situ* hybridization and immunocytochemistry. Under conditions of differentiation-induction with TPA, the expression of EGF or TGF-α mRNA was down-regulated and new expression of another growth factor was not detected in four MRT cell lines; TM87-16, STM91-01, TTC642 and YAM-RTK1. The down-regulated EGF or TGF-α rapidly recovered to the initial value in all MRT cell lines after changing the medium to one not containing TPA. Although TGF-α mRNA was found to be down-regulated in TTC549 with TPA induction, EGF mRNA appeared after induction with TPA and was subsequently down-regulated from day 4 to 6 of the treatment. *In situ* hybridization and double-staining immunocytochemistry confirmed this transient appearance of EGF.

To critically assess the autocrine loop of EGFR-EGF/TGF-α in MRT, we used mAbs against EGF, TGF-α and EGFR in the serum-free cell culture. Monoclonal antibodies against TGF-α and EGFR (EGFR-1), which inhibit the EGFR/TGF-α autocrine growth-regulatory mechanism, showed specific dose-dependent inhibitory effects on the proliferation of TM87-16, STM91-01 and TTC642. These results would suggest that the proliferation of these cell lines was regulated by an EGFR/TGF-α autocrine loop. Further, the finding that anti-EGF mAb did not have growth-inhibitory effects, indicated that EGFR/EGF was not a key mechanism for the proliferation of these cell lines.

In contrast, monoclonal antibodies against EGF and EGFR (EGFR-1), which antagonize the EGFR/EGF autocrine loop, showed a specific dose-dependent inhibition of the proliferation of YAM-RTK1 cells. One of the MRT cell lines, YAM-RTK1, was considered to be regulated by an EGFR/EGF autocrine loop.

TTC549, very interestingly, was found to have mRNA and protein of EGF newly expressed after differentiation-induction with TPA. Growth of TTC549 cells was significantly inhibited by mAb to EGFR alone. Moreover, simultaneous administration of anti-TGF-α and anti-EGF mAb was required to suppress proliferation of this cell line. These results would suggest that the autocrine loop of TGF-α/EGFR basically had a major role in the regulation of cell proliferation of TTC549, while the EGF/EGFR autocrine growth-regulatory pathway may play a substitutional role. This substitutional phenomenon was reversible. This compensatory autocrine mechanism was not found in any of the neuroblastoma cells or RD after treatment with TPA.

In summary, our results suggested that two distinct autocrine loops of EGFR-EGF and/or EGFR-TGF-α exist in MRT. Complementary autocrine growth regulatory loops may contribute to the more aggressive behavior of MRT. In addition, the presence of different autocrine loops in MRT cell lines would support the multiphenotypic diversity of this highly malignant neoplasm.

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